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VIRUS-CELL INTERACTIONS

Integrity of the Linker of Nucleoskeleton and Cytoskeleton Is Required for Efficient Herpesvirus Nuclear Egress

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ABSTRACT Herpesvirus capsids assemble in the nucleus, while final virion maturation proceeds in the cytoplasm. This requires that newly formed nucleocapsids cross the nuclear envelope (NE), which occurs by budding at the inner nuclear membrane (INM), release of the primary enveloped virion into the perinuclear space (PNS), and subsequent rapid fusion with the outer nuclear membrane (ONM). During this process, the NE remains intact, even at late stages of infection. In addition, the spacing between the INM and ONM is maintained, as is that between the primary virion envelope and nuclear membranes. The linker of nucleoskeleton and cytoskeleton (LINC) complex consists of INM proteins with a luminal SUN (Sad1/UNC-84 homology) domain connected to ONM proteins with a KASH (Klarsicht, ANC-1, SYNE homology) domain and is thought to be responsible for spacing the nuclear membranes. To investigate the role of the LINC complex during herpesvirus infection, we generated cell lines constitutively expressing dominant negative (dn) forms of SUN1 and SUN2. Ultrastructural analyses revealed a significant expansion of the PNS and the contiguous intracytoplasmic lumen, most likely representing endoplasmic reticulum (ER), especially in cells expressing dn-SUN2. After infection, primary virions accumulated in these expanded luminal regions, also very distant from the nucleus. The importance of the LINC complex was also confirmed by reduced progeny virus titers in cells expressing dn-SUN2. These data show that the intact LINC complex is required for efficient nuclear egress of herpesviruses, likely acting to promote fusion of primary enveloped virions with the ONM.

IMPORTANCE While the viral factors for primary envelopment of nucleocapsids at the inner nuclear membrane are known to the point of high-resolution structures, the roles of cellular components and regulators remain enigmatic. Furthermore, the machinery responsible for fusion with the outer nuclear membrane is unsolved. We show here that dominant negative SUN2 interferes with efficient herpesvirus nuclear egress, apparently by interfering with fusion between the primary virion envelope and outer nuclear membrane. This identifies a new cellular component important for viral egress and implicates LINC complex integrity in nonconventional nuclear membrane trafficking.

KEYWORDS herpesvirus, pseudorabies virus, nuclear egress, nuclear envelope, linker of nucleoskeleton and cytoskeleton, LINC complex, SUN2

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Herpesvirus nucleocapsids assemble in the nucleus, while further maturation to infectious particles occurs in the cytoplasm. The nucleus, the defining feature of eukaryotic cells, is surrounded by two concentric lipid bilayers, designated the inner nuclear membrane (INM) and the outer nuclear membrane (ONM). The two membranes are joined at annular junctions harboring the nuclear pore complexes. While these

nuclear pore complexes allow the import of the viral genome during initiation of infection, they are too small for translocation of the newly formed, approximately 125-nm nucleocapsid. Instead, export of mature nucleocapsids is mediated by vesicles derived from the INM which fuse with the ONM in a well-tuned but molecularly still enigmatic process. This step rapidly and efficiently releases nucleocapsids into the cytoplasm (reviewed in reference 1).

Conserved herpesviral proteins, designated pUL34 and pUL31 in the porcine alphaherpesvirus pseudorabies virus (PrV) and the human alphaherpesviruses herpes simplex virus 1 (HSV-1) and HSV-2, form the nuclear egress complex (NEC). They are required for efficient nuclear egress and also define the minimal unit for budding and scission of vesicles from the INM (2, 3). However, it appears that the NEC is insufficient for fusion with the ONM, since coexpression of pUL31 and pUL34 in the absence of viral infection causes vesicles to accumulate in the perinuclear space (PNS) within large invaginations of the INM (2, 3). These data also highlight that primary envelope fusion with the ONM is an active process that does not innately takes place once vesicles reach the PNS. The viral pUS3 kinase appears to have a role in ONM fusion, since deletion or inactivation causes primary virions to accumulate within INM invaginations (4–6).

It is also still unclear whether membrane fusion during nuclear egress involves the same viral glycoproteins that mediate fusion of the mature virion envelope with the host cell plasma membrane during entry (7–9). While for HSV-1 simultaneous deletion of glycoproteins gB and gH, which are the major constituents of the herpesvirus core fusion machinery, causes primary virions to accumulate in the PNS, consistent with their participation in deenvelopment (10), nuclear egress of PrV was normal in the absence of gB and/or gH or other viral glycoproteins, singly or in different combinations (11), strongly arguing against a function in deenvelopment. In addition, viral glycoproteins could not be detected in the INM of PrV-infected cells or in primary virions by immunoelectron microscopy (11). The failure to identify crucial viral factors suggests that cellular proteins are responsible for fusion of the primary virion envelope and the ONM. Indeed, it appears that fusion events are a normal occurrence at the nuclear membranes. Nuclear membrane remodeling and fusion are involved in postmitotic reassembly of the nuclear envelope, interphase nuclear pore insertion, and trafficking of ribonucleoprotein particles out of the nucleus (12, 13). However, while the mechanisms underlying nuclear membrane fusion are under intensive investigation (reviewed in reference 14), little is known about fusion machineries or factors that promote or restrict fusion at this cellular compartment.

The INM and ONM are coupled by the linker of nucleoskeleton and cytoskeleton (LINC) complex (15). The LINC complex is formed by KASH ((Klarsicht, ANC-1, SYNE homology) domain proteins located in the ONM, which extend into the cytosol to contact cytoskeletal filaments, and SUN (Sad1/UNC-84 homology) domain proteins embedded in the INM that anchor to the nuclear lamina. SUN and KASH proteins tightly interact in the PNS to form the nuclear envelope bridging complex (16, 17). The LINC complex positions the nucleus in the cell, is critical for nucleo-cytoskeletal force transmission (18), and acts as a molecular spacer that maintains a constant perinuclear distance of ca. 30 to 50 nm between the nuclear membranes (19–21).

SUN proteins consist of a variable N-terminal nucleoplasmic domain followed by a transmembrane domain and a coiled-coil region located in the PNS. The majority of the remaining perinuclear region is the so-called SUN domain, named according to its homology with domains first described in *S*ad1 from *Schizosaccharomyces pombe* and *UN*C-84 from *Caenorhabditis elegans* (reviewed in reference 19). The molecular structure of the LINC complex has been solved. It consists of a trimer of SUN domains bound to a trimer of KASH peptides within the PNS that forms a molecular lattice (19). However, the picture of a simple SUN-KASH function is challenged by the detection of an increasing number of interaction partners and regulators (22). Moreover, SUN proteins in particular are implicated in a pleiotropic set of functions, including nuclear membrane reorganization (23, 24).

The spacing role attributed to the LINC complex and the implication that it may

affect membrane remodeling raised our interest in whether an intact LINC complex affects nuclear membrane fusion events in herpesvirus nuclear egress requiring efficient translocation of viral particles from the nucleus into the cytosol. During human cytomegalovirus infection, the tethering between the INM and ONM is lost, probably due to a decrease in levels of SUN1 and SUN2 (25). In striking contrast, numerous studies performed in our laboratory with PrV and HSV-1, in different cell lines and after different time points of infection, showed that the distance between the INM and ONM, and also that between the primary virion envelope and the adjacent nuclear membrane, is maintained even late after infection. This indicates that the LINC complex is stable during infection. Different scenarios can thus be envisioned: (i) the LINC complex lattice within the PNS has to be dislocated or dissociated to accommodate the approximately 140-nm primary virion; (ii) the LINC complex is passively involved, keeping the primary virions at a "fusion-compliant" distance from the ONM; or (iii) the LINC complex is actively involved in nuclear egress by, e.g., generating forces onto and pulling the primary virion envelope toward the ONM to facilitate fusion.

To test for a function of the LINC complex during nuclear egress, we overexpressed dominant negative (dn), soluble luminal (lu) forms of the SUN components that compete with the normal perinuclear bridging to produce nonanchored LINC complexes. Overexpression of dn-SUN1 and dn-SUN2 resulted in an expansion of the PNS in noninfected rabbit kidney (RK13) cells. After infection of dn-SUN2-expressing cells with PrV, the PNS was tremendously enlarged and primary enveloped virions accumulated in outward bulges of the ONM and were also detected in intracytoplasmic lumina, most likely the endoplasmic reticulum (ER). This was never observed in nontransgenic cells, where only single primary virions in the PNS are observed. In addition, titers of wild-type PrV derived from the dn-SUN2-expressing cell line were reduced. These data show that SUN domain proteins support nuclear egress of herpesviruses, appearing to act at the stage of primary virion envelope fusion with the ONM.

RESULTS

Maintenance of spacing between the nuclear membranes during infection. In our numerous infection experiments we consistently observed that spacing between the INM and ONM remains largely unaltered during infection with PrV and HSV-1 on cell lines derived from different species. Although the PNS has to accommodate the approximately 140-nm primary enveloped virions, the distance between the INM and ONM or between the primary virion envelope, which is derived from the INM, and the nuclear membranes appears to be constant (approximately 28 nm $\pm$ 8 nm) (Fig. 1). This suggests that the LINC complex must remain intact and functional during herpesvirus infection and nuclear translocation of virions.

Luminal forms of SUN1 and SUN2 localize to the nuclear envelope. The luminal domains of SUN1 and SUN2 have been shown to act as dominant negative (dn) forms disturbing the LINC complex and the spacing between the nuclear membranes (15). To test whether this is also evident in rabbit kidney (RK13) cells, plasmids carrying the luminal, dominant negative forms of SUN1 and SUN2 (Fig. 2A) were expressed and analyzed directly by green fluorescent protein (GFP) autofluorescence. As shown in Fig. 2B, both proteins showed a clear nuclear rim staining, indicating correct targeting. lu-SUN1 exhibited, in addition, an ER-like staining pattern, as reported previously (15).

Overexpression of luminal SUN proteins results in an expansion of the perinuclear space. To test whether the LINC complex controls nuclear membrane spacing during PrV infection, we generated RK13 cell lines that stably express the luminal domains of SUN1 and SUN2. The relative expression in the cell lines was tested by immunoblotting (Fig. 2C) using either anti-GFP, anti-SUN1, or anti-SUN2 specific rabbit antisera. The anti-GFP serum detected ca. 80-kDa major proteins in both cell lines, with stronger signals in the lu-SUN1-expressing cells than in the lu-SUN2-expressing cells. The SUN1- or SUN2-specific antisera reacted most strongly with the homologous antigen, as expected. Endogenous rabbit SUN1 and SUN2, which are predicted to have molecular masses similar to those of the GFP-tagged luminal forms, were only weakly

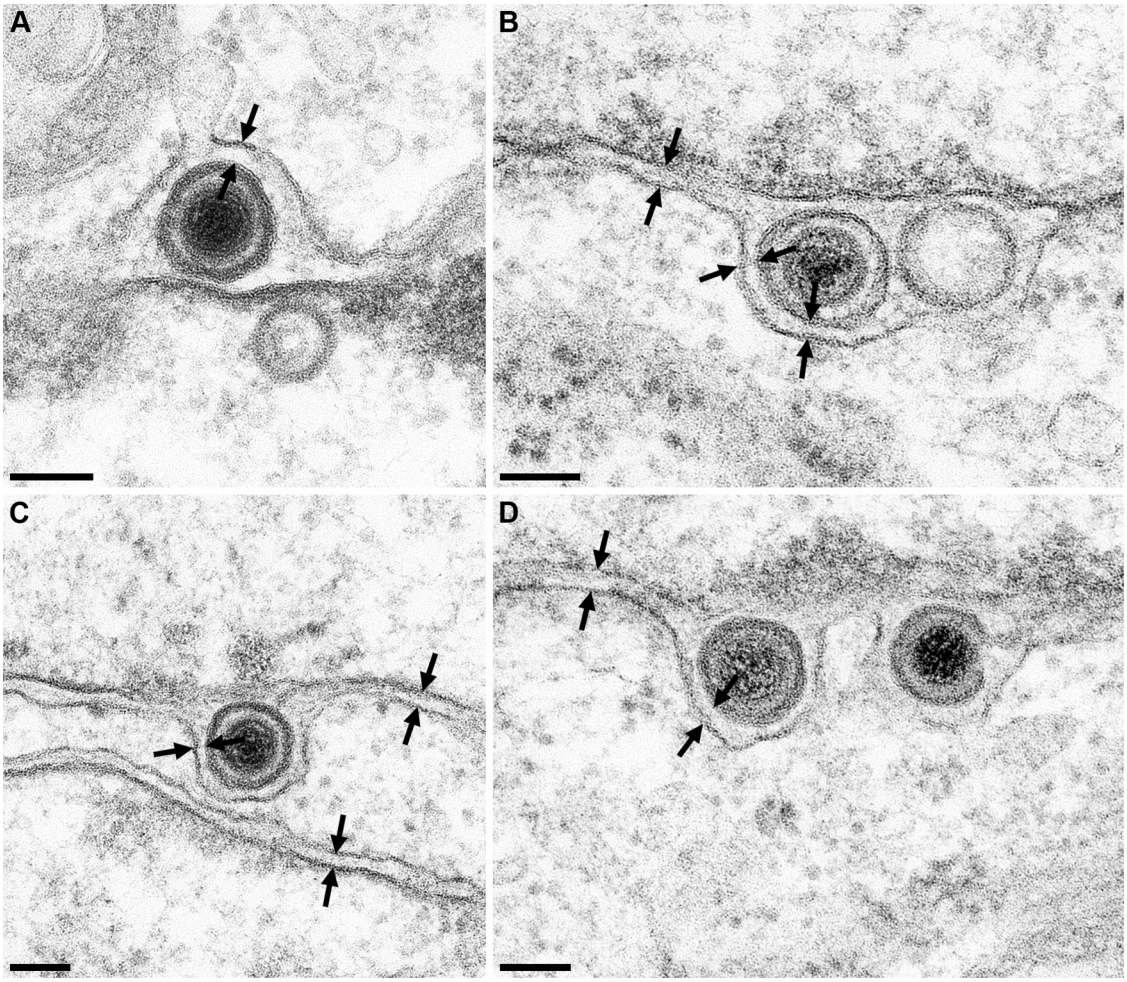

FIG 1 Primary virions in the perinuclear space after PrV infection. RK13 cells were infected with PrV-Ka and processed for electron microscopy at 14 h p.i. Representative images of primary enveloped virions in the perinuclear cleft are shown. Arrows indicate similar spacing between the different membranes. Bars, 100 nm.

detected. Proteins of smaller size may represent degradation products visible because of the overexpression, but their origin remains unclear at present.

To test whether lu-SUN overexpression affects the nuclear envelope, we used electron microscopy to examine RK13-GFP-lu-SUN1 and RK13-GFP-lu-SUN2 cells as well as nontransgenic RK13. As shown in Fig. 3, cells expressing GFP-lu-SUN1 (Fig. 3B) showed a slight expansion of the perinuclear cleft and the endoplasmic reticulum compared to nontransgenic RK13 cells (Fig. 3A). However, RK13-GFP-lu-SUN2 cells exhibited a significantly enlarged PNS, filled with electron-dense material, which was also found in the dilated intracytoplasmic lumen, most likely ER (Fig. 3C). The distances were measured in 12 cells, each in three different sections derived from two different preparations. The results are summarized in Fig. 3D. While the mean distance between the two membranes in normal RK13 cells was 22.7 nm, the spacing was more than double that in RK13 cells expressing GFP-lu-SUN2 (52.5 nm), while it was intermediate in cells expressing GFP-lu-SUN1 (36.1 nm).

Overexpression of GFP-lu-SUN2 causes primary enveloped virions to accumulate in the PNS and ER lumen. Cells overexpressing the luminal forms of SUN1 and SUN2 were infected with the wild-type strain PrV-Kaplan (PrV-Ka) at a multiplicity of infection (MOI) of 1 and processed for electron microscopy at 14 h postinfection (p.i.). No obvious defect was seen after infection of cells expressing GFP-lu-SUN1 (Fig. 4A). In contrast, the GFP-lu-SUN2-expressing cells accumulated primary enveloped virions,

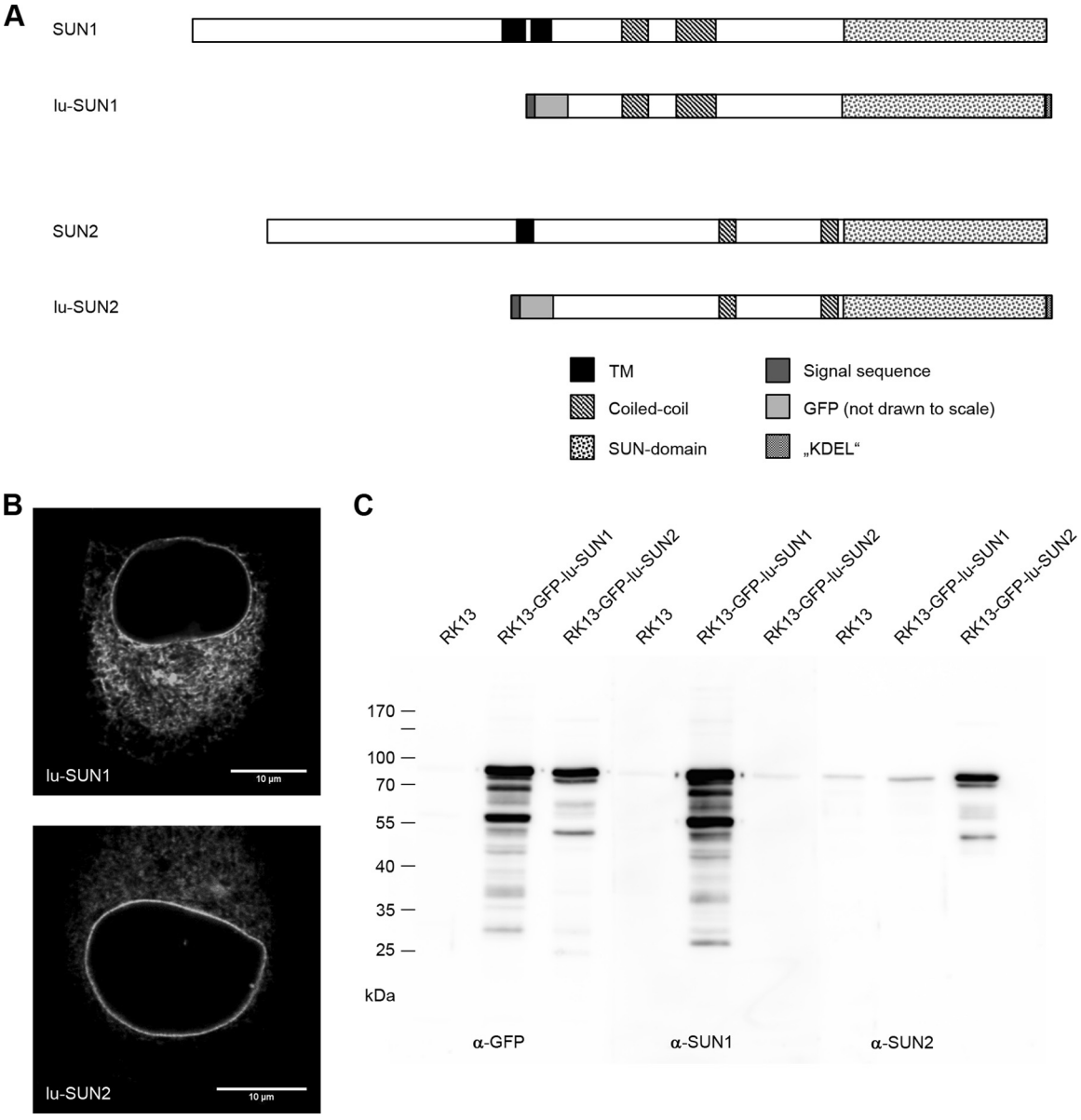

FIG 2 Expression of lu-SUN1 and lu-SUN2 proteins. (A) Schematic representation of expressed proteins used in this study. Expression products for the luminal domains of SUN1 and SUN2 comprise an amino-terminal GFP tag, a signal sequence for targeting into the ER and PNS, and the ER retention signal KDEL at the C terminus to prevent secretion. (B) RK13 cells were transfected with expression plasmids for the GFP-tagged luminal domains of SUN1 and SUN2. Cells were fixed with paraformaldehyde 2 days after transfection, and GFP autofluorescence was detected with a laser scanning microscope. (C) Stably GFP-lu-SUN1- and GFP-lu-SUN2-expressing cell lines were harvested, and cellular lysates were separated on an SDS-10% polyacrylamide gel. Blots were probed with antisera specific for GFP, SUN1, and SUN2 as indicated. Molecular masses of marker proteins (in kDa) are given on the left.

identified by the smooth virion envelope and the condensed virion tegument (7, 8), in the dilated PNS and bulges of the ONM (Fig. 4B and C). These were only rarely observed in PrV-Ka-infected wild-type cells and are a strong indicator that the efficiency of viral egress is reduced when LINC complex integrity is compromised. Indeed, primary enveloped virions were also present in a lumen at a significant distance from the nucleus, suggesting that they diffuse out of the PNS into the main ER network (Fig. 4C, asterisk). The topography of primary virion accumulation in dn-SUN2 cells also appeared to be different from that described upon infection with PrV-ΔUS3 (4, 5). The dn-SUN2 cells lacked the INM herniations that deflect into the nucleus, and instead they produced bulges of the ONM into the cytoplasm. We then also examined the nuclear envelope morphology of dn-SUN1 and dn-SUN2 cells infected with PrV-ΔUS3 (Fig. 5).

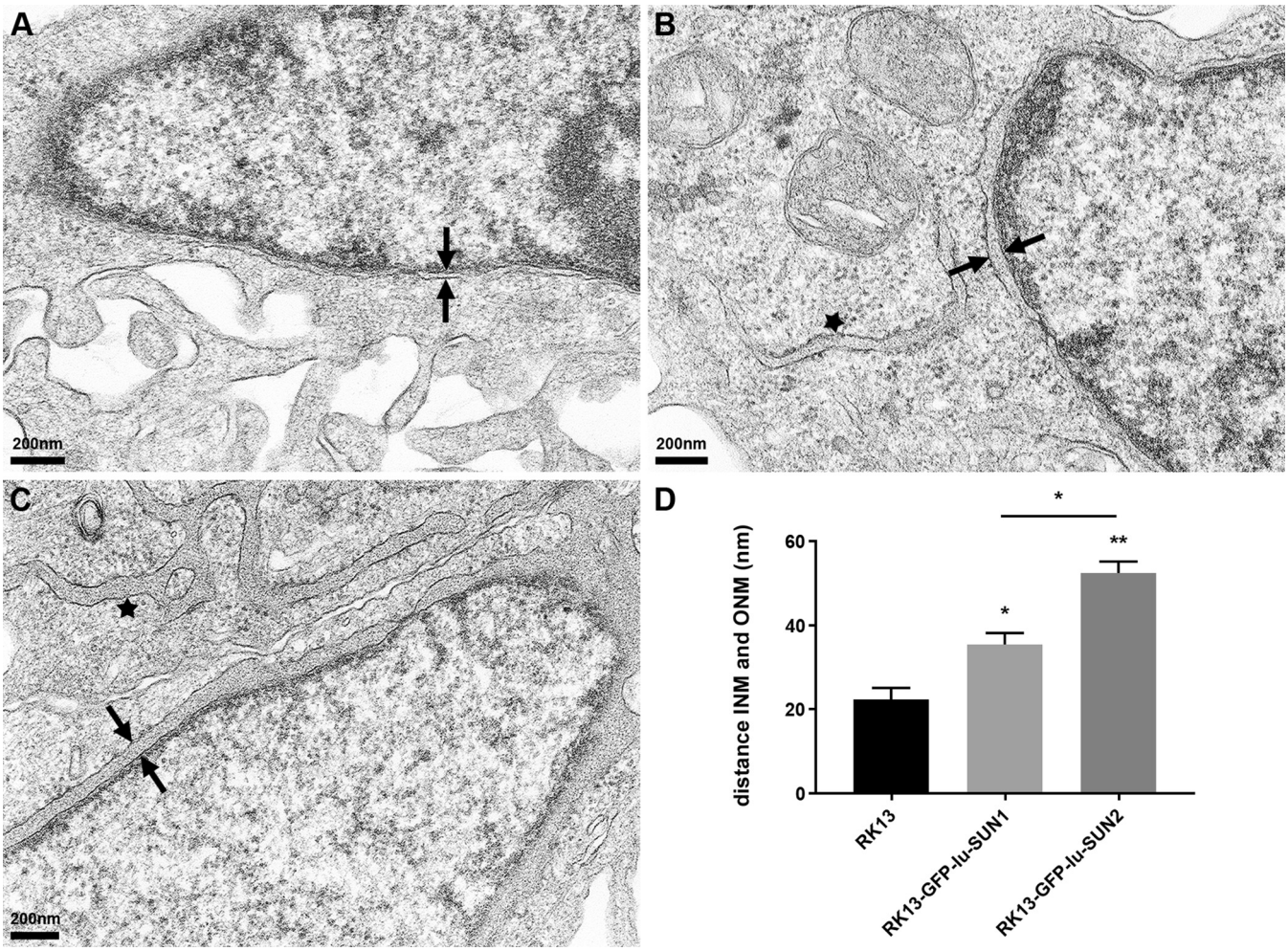

FIG 3 Effect of overexpression of luminal domains of SUN1 and SUN2 on the nuclear envelope. (A to C) RK13 cells (A) and RK13 cells stably expressing GFP-lu-SUN1 (B) or GFP-lu-SUN2 (C) were fixed and processed for electron microscopy. Different spacing of the INM and ONM is indicated by arrows, and dilated ER is marked by an asterisk. Bars, 200 nm. (D) The distance between INM and ONM was measured in 12 cells each derived from three different sections and two independent preparations. The mean value and corresponding standard deviation are shown, and statistically significant differences are indicated (*, $P \leq 0.05$; **, $P \leq 0.01$).

This caused the well-known PrV-ΔUS3 invaginations of the INM filled with primary virions (4, 5) in RK13 and RK13-GFP-Lu-SUN1 cells, in addition to the ONM bulges with accumulated primary virions due to impairment of the LINC complex only in cells expressing GFP-lu-SUN2. Apparently, both phenotypes can occur independently of each other at the same nuclear envelope, indicating that pUS3 and the LINC complex function independently of each other in viral egress.

Overexpression of GFP-lu-SUN2 results in decreased viral titers. To test whether the enlargement of the PNS and the accumulation of virions in the perinuclear cleft and the ER lumen influence viral titers, RK13, RK13-GFP-lu-SUN1, and RK13-GFP-lu-SUN2 cells were infected with PrV-Ka and harvested at 24 h p.i. As shown in Fig. 6, titers produced after infection of RK13-GFP-lu-SUN2 were approximately 10-fold lower than those from nontransfected cells. Approximately 10-fold-lower viral titers were also found after infection with PrV-ΔUS3 from RK13 cells, as described previously (5), but the titers were not further reduced on RK13-GFP-lu-SUN2 cells (data not shown).

DISCUSSION

Herpesvirus replication depends on efficient translocation of virions from the nucleus across the two nuclear membranes into the cytosol. While envelopment of nucleocapsids at the INM is elucidated to some extent, it is still unclear how fusion of

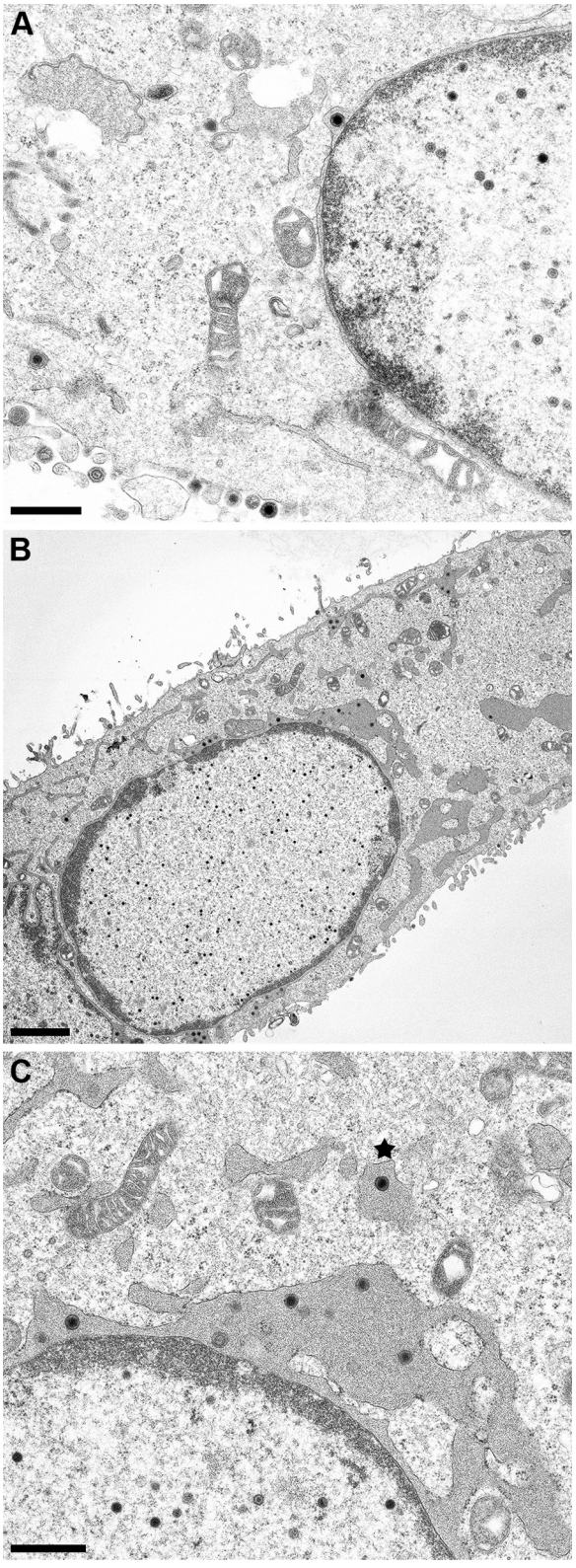

FIG 4 Overexpression of GFP-lu-SUN2 results in accumulation of primary enveloped virions in the perinuclear space and escape into the ER. RK13-GFP-lu-SUN1 (A) or RK13-GFP-lu-SUN2 (B and C) cells were infected with PrV-Ka at an MOI of 1 for 14 h. Panel C is a higher magnification of the infected RK13-GFP-lu-SUN2 cell depicted in panel B, showing primary virions in the dilated perinuclear cleft and in the ER (marked by asterisk). Bars, 700 nm in panels A and C and 2 μm in panel B.

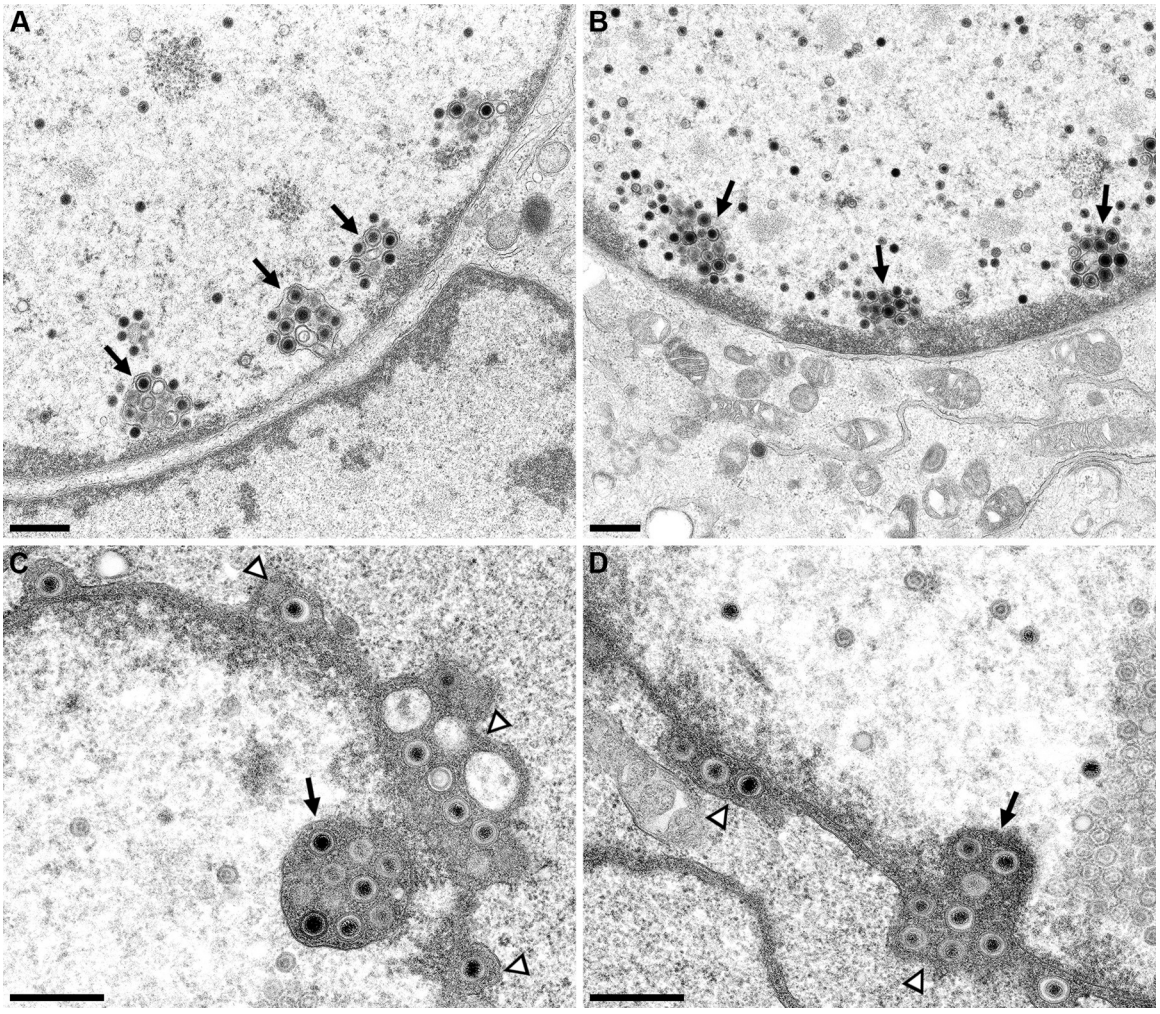

FIG 5 Herniations of INM and ONM after infection of RK13-GFP-lu-SUN2 cells with PrV-ΔUS3. RK13 (A), RK13-GFP-lu-SUN1 (B), and RK13-GFP-lu-SUN2 (C and D) cells were infected with PrV-ΔUS3 at an MOI of 1 for 14 h. The representative images show invagination of the INM into the nucleoplasm (arrows) in RK13, RK13-GFP-lu-SUN1, and RK13-GFP-lu-SUN2 cells as well as bulging of the ONM into the cytosol (arrowheads) filled with primary enveloped particles, which are detectable only in RK13-GFP-lu-SUN2 cells. Bars, 500 nm.

the primary virion envelope with the ONM is achieved and how it is regulated to preserve nuclear membrane integrity during infection.

Here we targeted cellular proteins that may contribute to viral egress after observing that the membrane spacing between the INM and ONM and between the primary envelope and both nuclear membranes remains similar even when viral nucleocapsids are translocating across the nuclear membranes. This suggested that the cellular machinery that spaces the membranes, the LINC complex, remains intact during viral egress. This is somewhat surprising considering the membrane scission and fusion events taking place and the expectation that dissolution of the LINC complex lattice that fills the PNS would promote efficient viral trafficking. The fact that the spacing remained unaltered instead suggested that, in fact, an intact LINC complex supports viral egress. This is an interesting possibility since elements of the LINC complex have been implicated in remodeling of the nuclear membranes during nuclear pore biogenesis (23).

Therefore, we tested the involvement of the LINC complex in herpesvirus replication. SUN proteins are the transmembrane INM elements of the complex that have a nuclear N terminus that binds the nuclear lamina and a luminal C terminus that binds KASH proteins in the PNS. In turn, KASH proteins span the ONM to connect with cytoskeletal elements in the cytosol (26). We disrupted the complex using a previously

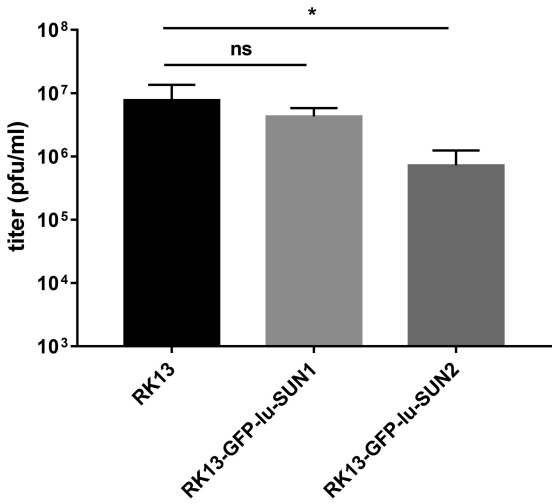

FIG 6 One-step growth kinetics. RK13, RK13-GFP-lu-SUN1, and RK13-GFP-lu-SUN2 cells were infected with PrV-Ka and harvested at 24 h p.i. Progeny titers were determined on RK13 cells. Shown are mean values from three independent experiments and corresponding standard deviations. A statistically significant difference is indicated (*, $P < 0.05$; ns, nonsignificant).

described strategy of expressing the isolated luminal domains of SUN proteins. These dominant negative (dn) fragments compete against the endogenous transmembrane SUN proteins for KASH protein binding (15). They also achieve LINC complex disruption without removing native SUN proteins from the INM, where these proteins have roles in addition to those within the LINC complex (23, 24, 27, 28).

We find that the dn-SUN fragments increase nuclear membrane spacing, as has been previously reported (15). However, in RK13 cells, dn-SUN2 was significantly more effective than dn-SUN1, more than doubling the distance between INM and ONM compared to that in nontransgenic RK13 cells. The reason why dn-SUN2 activity is more prominent than dn-SUN1 is unclear. It appears to be unrelated to expression levels, since higher levels of dn-SUN1 than dn-SUN2 expression were actually detected in the stably SUN-overexpressing cell lines. Simultaneous expression of dn-SUN1 and dn-SUN2 had no additional effect on nuclear membrane spacing (data not shown). Therefore, it may instead relate to which KASH proteins are expressed by RK13 cells and their relative affinity for SUN1 versus SUN2. The fact that dn-SUN1 and dn-SUN2 expression is sufficient to expand the PNS shows that either protein effectively disturbed the LINC complex, a prerequisite for analysis of the importance of an intact LINC complex for viral egress. Indeed, enlarged nuclear envelope spacing was also observed after viral infection. The increase in spacing correlated with the accumulation of primary virions in the PNS, which is rarely observed due to the efficient nature of viral egress. There were some primary virions evident in the slightly enlarged PNS of cells expressing dn-SUN1, although virus titers derived from these cell lines were comparable to those from nontransgenic RK13 cells. However, after PrV infection of dn-SUN2 cells, the expansion of the PNS was extreme. Here primary virions, easily discernible by the smooth virion envelope and the electron-dense compact tegument (7, 8), were found within the drastically dilated PNS. In addition, multiple primary enveloped virions were observed even at a great distance to the nucleus within membranous organelles, which most likely are derived either from the expanded PNS or from connected ER. Although the ONM, the ER membrane, and, thereby, also the corresponding lumina are continuous, deenvelopment of the primary virus particles occurs predominantly via fusion with the ONM. Primary virions distant from the nucleus within the ER are only very rarely detected after infection of normal cultured cells with wild-type PrV, indicating that a physical barrier hampers escape of the primary virion into the ER. Alternatively, fusion with the ONM may occur more rapidly than virion diffusion from this zone. However, after expression of GFP-lu-SUN2, numerous virions were detected in tubular

structures in the cytoplasm, even at a great distance from the nucleus, indicating that the restriction of the primary virions to the PNS is lost in the presence of dn-SUN2. The accumulations seen in electron microscopic images correspond well with the observed approximately 10-fold reduction in viral titers derived from RK13-GFP-lu-SUN2 cells compared to those from nontransgenic RK13 cells.

The phenotype of infected dn-SUN2 cells, where primary virions accumulate in a PNS that expands out into the cytosol, strongly suggests that an intact LINC complex supports efficient ONM fusion. Unfortunately, immunolabeling experiments to detect SUN proteins in primary virions or the nuclear membrane were unsuccessful (data not shown), which might be due to the weak reactivity of the available sera or the limited abundance of the antigen. We also examined the relationship between this newly uncovered LINC-dependent aspect of viral egress and that facilitated by the pUS3 kinase. Loss of pUS3 kinase also hampers viral egress and traps virions within the PNS, but in this case they accumulate in invaginations of the INM protruding into the nucleoplasm (4–6). We were interested in whether the two phenotypes differ based on whether the LINC complex is intact (invaginations into the nucleoplasm) or disrupted (protrusions into the cytosol and virions located distant from the PNS). Surprisingly, we found both types of defects when we infected dn-SUN2 cells with PrV-ΔUS3. It is not clear why virions still accumulate adjacent to the INM when dn-SUN2 has greatly expanded the PNS. One possible explanation is that pUS3 contributes to INM scission and thus helps release virions from the INM into the PNS. In this scenario, the ΔUS3 phenotype reflects virions that are stalled while still attached to the INM, causing it to bulge into the nucleoplasm. Once virions detach from the INM, they then accumulate in the expanded PNS since dn-SUN2 expression reduces the efficiency of ONM fusion and allows outward expansion of the PNS.

Our data point to a role for the LINC complex in ONM fusion during viral egress. However, they do not distinguish whether the LINC complex plays an active role or assists viral egress by, e.g., trapping primary virions within the PNS and/or maintaining close apposition between the INM and ONM for efficient membrane trafficking. In support of an active role, it seems surprising that an increased distance of only ~30 nm between the INM and ONM impairs viral trafficking given that cytosolic vesicle trafficking is efficient over much longer distances. Thus, while this is speculative, we propose that the LINC complex may actively contribute to trafficking, acting at a stage after primary virions are released from the INM into the PNS. We also base this on the fact that dn-SUN2 expression not only affects PNS spacing but also disturbs the usually very consistent, stereotyped spacing that exists between virions and the nuclear membranes.

Primary virions and virus-like particles within the lumen of the ER were also described previously after HSV-1 infection of neuron-like PC6-3 cells overexpressing torsinA (29). Torsins are members of the AAA+ (ATPases associated with a variety of cellular activities) ATPase superfamily which localize to the lumen of the ER and the PNS (reviewed in reference 30). Torsins are implicated in constitutive cellular nuclear vesicle trafficking, since their deletion produces characteristic INM abnormalities that closely resemble vesicles that budded but failed to undergo scission from this membrane (31), and torsin loss may inhibit the trans-nuclear membrane trafficking of ribonucleoprotein particles (32). However, the predominant model of torsin function is that they help release vesicles from the INM into the PNS, in which case it is difficult to understand why torsinA overexpression causes virions to accumulate in the ER lumen (29), a phenotype that instead points to a deficit in ONM fusion. Our data now suggest a mechanism to resolve this issue. TorsinA is also strongly implicated in LINC complex regulation, including removing LINC complexes from the nuclear envelope and especially affecting those containing SUN2 (33). Thus, torsinA or dn-SUN2 overexpression may similarly cause virions to accumulate in the ER lumen because they converge to disrupt endogenous SUN2-containing LINC complexes. Also interesting is the fact that overexpression of torsinA in neuron-like PC6-3 cells (29) or overexpression of dn-SUN2 in epithelial RK13 cells (this study) resulted in a 5- to 10-fold decrease in viral titers. This

is most likely due to the escape of primary virions into the ER, a membrane system which might not be competent to allow efficient deenvelopment.

In summary, our data show that an intact LINC complex promotes viral egress, acting at a stage after primary virion scission from the INM. Future work should examine whether the LINC complex plays a passive or rather active role during fusion of the primary envelope with the ONM. It will also be interesting to elucidate whether the same mechanism contributes to normal nuclear membrane remodeling events that occur independently from herpesviral infection.

MATERIALS AND METHODS

Cells and viruses. Rabbit kidney (RK13) cells were cultivated in minimum essential medium with 10% fetal calf serum at 37°C and 5% CO_2. PrV strain Kaplan (PrV-Ka) (34) or PrV-ΔUS3 (5) was used for infection studies. Viruses were propagated in RK13 or pig kidney cells.

Expression constructs and antisera. Plasmids expressing the luminal forms of SUN1 or SUN2 were cloned into vector derived from pcDNA3.1 V5/HIS-TOPO (Invitrogen). SUN1 sequence was amplified from Mammalian Gene Collection (MGC) clone 61309 (BC_048156) using primers 5′-CAG AGG GTG GAC GAT TCC AAG-3′ and 5′-CTG GAT GGG CTC TCC GTG GA-3′. SUN2 sequence was amplified from clone DKFZp686F10107 (BX_537962) with upper primer 5′-AGC AGG AGG CCG GAT GAG GG-3′ and lower primer 5′-GTG GGC GGG CTC CCC ATG CA-3′. In both cases, upper primers contained a NheI site and lower primers contained a SpeI site, and PCR products were initially cloned into pCRII (Invitrogen). Nucleotide sequences encoding KDEL and a stop codon were then inserted as oligomers into the terminal SpeI site to retain protein fragments within the ER. Then, the ER-targeting signal sequence of human calreticulin (ATGCTGCTATCCGTGCCGT to CCTCGGCCTGGCCGTCGCC derived from DsRed-ER [Clontech]) was cloned into the NheI sites while retaining them. The GFP sequence from pEGFP-N1 (Clontech) (where ATG and TGA codons were altered to NheI and SpeI) was subsequently cloned into the same NheI sites. The GFP-tagged SUN1 and SUN2 fragments that lack transmembrane regions (modeled on those described in reference 15) (Fig. 2A) were then recloned into the mammalian expression vector pcDNA3.1 V5/HIS-TOPO. The full-length forms were used as controls.

Transfection and infection experiments. For generation of stably SUN protein-overexpressing cell lines, RK13 cells were transfected with the expression constructs for GFP-lu-SUN1 and GFP-lu-SUN2. Cell clones were selected in medium containing 500 μg/ml G418 (Invitrogen) and picked by aspiration. Expression was tested by direct visualization of GFP autofluorescence and by immunoblotting.

Confocal microscopy. For confocal microscopy, RK13 cells were transfected with the expression constructs for GFP-lu-SUN1 and GFP-lu-SUN2, and expressed proteins were directly visualized after fixation with 3% paraformaldehyde by autofluorescence. Images were obtained using a confocal laser scanning microscope (SP5; Leica, Germany).

Immunoblotting. Stably expressing cell lines were harvested by scraping into the medium, washed with phosphate-buffered saline (PBS), and lysed in SDS sample buffer (0.13 M Tris-HCl [pH 6.8], 4% SDS, 20% glycerin, 0,01% bromophenol blue, 10% mercaptoethanol). Proteins were separated in SDS-10% polyacrylamide gels, and blots were probed with anti-GFP (kindly provided by G. M. Keil, FLI, Insel Riems, Germany), anti-SUN1, and anti-SUN2 sera. Polyclonal anti-SUN1 and anti-SUN2 rabbit sera were raised against purified recombinant glutathione *S*-transferase (GST)-tagged luminal domains of mouse SUN1 (NM_024451; codon 458 to stop codon at position 931) and SUN2 (NM_001205345; codon 276 to stop codon at position 731) expressed from pDEST15 (Invitrogen) in *Escherichia coli* BL21.

Electron microscopy. For ultrastructural examination, cells were either mock infected or infected with PrV-Ka or PrV-ΔUS3 at a multiplicity of infection (MOI) of 1 for 14 h. Cells were fixed and processed for electron microscopy as described previously (35). The distance between the INM and ONM was measured for 12 cells in three different slices derived from two independent preparations. Statistically significant differences were analyzed using the ordinary one-way analysis of variance (ANOVA) followed by the Tukey multiple-comparison test as provided by GraphPad Prism 7 software (P values of <0.05 were considered significant).

***In vitro* replication studies.** RK13 cells or RK13 cells overexpressing the dominant negative LINC complex component lu-SUN1 or lu-SUN2 were infected with PrV-Ka or PrV-ΔUS3 at an MOI of 5 and harvested 24 h later. Progeny titers were determined on RK13 cells. Mean values from three independent assays were calculated and plotted with the corresponding standard deviation. Statistics were evaluated using the unequal-variance t (Welsh) test provided by GraphPad Prism 7 software (a P value of <0.05 was considered significant).

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